Remarks

Claims 1, 4, 18 and 20 have been amended without abandonment or prejudice to Applicants' rights to pursue any canceled subject matter in one or more continuing applications.

Claim 1 has been amended to (a) restrict the heterocyclic group recited in R^4 to thienyl; (b) restrict the scope of R^3 by restricting R^5 to C_{1-6}alkyl and E to a direct bond; (c) remove a heterocyclic group as being a permissible variable for R^5, R^6 and R^7, which coupled with the amendment in (a), results in the elimination of R^8 and R^{11}; and (d) correct simple typographical errors.

Claim 4 has been amended to be consistent with amended claim 1.

Claim 18 has been amended to restrict the ischaemia to be treated to myocardial and cerebral ischaemia.

Claim 20 has been amended to correct simple typographical errors.

After entry of the above amendments, claims 1, 2, 4-10 and 16-21 will be pending.

No new matter has been introduced by any of the claim amendments.

Applicants note that claim 8 is objected to as being dependent upon a rejected base claim, but that the Examiner would find claim 8 allowable if rewritten in independent form including all of the limitations of the based claim and any intervening claims.

I. Rejection under 35 U.S.C. § 112, first paragraph

(a) Treatment of Ischaemia

Claim 18 is rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to a skilled artisan that the inventors, at the time the subject application was filed, had possession of the claimed invention. The Examiner objects to claim 18 being directed to treating all forms of ischaemias when, according to the Examiner, Applicants' specification only describes the treatment of myocardial ischaemia. The Examiner states that claim 18 would no longer be rejected if it were limited to treating myocardial ischaemia.

While the Applicants do not agree with the Examiner's assessment that the scope of method of treatment claim 18 is sufficiently encompassing as not to comply with the written

description requirement, Applicants have, in order to expedite prosecution of this application, restricted the ischaemia to be treated to myocardial ischaemia and cerebral ischaemia.

The Examiner errs when asserting that only myocardial ischaemia is described in the specification. Cerebral ischaemia finds support, for example, at page 29, line 31.

Applicants reserve the right to pursue in a future application any subject matter that is deleted by this restriction of claim 18. In light of the amendment of claim 18, Applicants request that the grounds for this rejection be withdrawn and that claim 18 be found in condition for allowance.

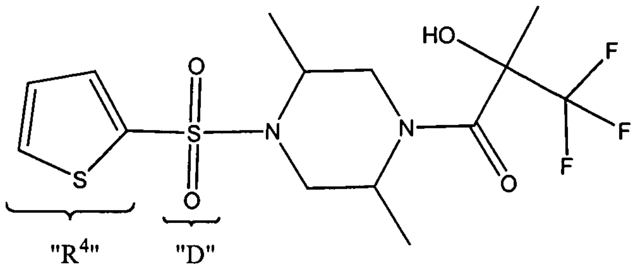
(b) Heterocyclic Groups and R^3

Claims 1, 2, 4-7, 9-10 and 16-21 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable a skilled artisan to make and/or use the invention. In particular, the Examiner contends that Applicants do not describe any representative examples encompassing any heterocyclic species and therefore objects to the recitation of a "heterocyclic group" as a possible variable for several of the "R" substituents of formula (I). Similarly, the Examiner objects to the scope of permissible choices for "R^3."

Applicants do not agree with the Examiner's assessment of claims 1, 2, 4-7, 9-10 and 16-21 as lacking enablement. Nevertheless, Applicants have, in order to expedite prosecution of this application, made the following amendments to claim 1 which further restrict the claimed subject matter relating to heterocyclic groups and the scope of R^3:

- removed a heterocyclic group as being a permissible variable for R^5, R^6 and R^7;
- restricted the heterocyclic group recited in R^4 to thienyl; and
- restricted R^5 to C_{1-6}alkyl and E to a direct bond (impacting the scope of R^3)

Contrary to the Examiner's assertion, Example 3 at page 32 of the specification does describe the preparation of the following heterocyclic group-containing compound (stereochemistry not indicated):



compound of Example 3

By restricting a heterocylic group as a permissible variable to R^4 only, and then further restricting the R^4 heterocyclic group to thienyl (which has the effect of completely eliminating both R^8 and R^{11}), Applicants believe that they have fully addressed the Examiner's rejection regarding the use of heterocylic groups.

Similarly, Applicants believe that by restricting R^5 to C_{1-6}alkyl and E to a direct bond, they have significantly reduced the scope afforded to R^3, thus fully addressing the Examiner's rejection regarding the same.

Applicants reserve the right to pursue in a future application any subject matter that is deleted by the aforementioned restrictions to the scope of claim 1. In light of these amendments to claim 1, Applicants request that the grounds for this rejection be withdrawn and that claims 1, 2, 4-7, 9-10 and 16-21 be found in condition for allowance.

(c) Method of Treatment Claims

Claims 18-20 are rejected under 35 U.S.C. § 112, first paragraph, as being not enabled. The Examiner asserts that Applicants have not supplied any competent evidence of record associating the disease states recited in claims 18-20 with elevation of PDH activity.

The Examiner acknowledges the reference cited in Applicants' specification that associates the elevation of PDH activity with the treatment of Alzheimer's disease (as previously pointed out to the Examiner in the filed response of April 26, 2004), but considers the reference to be speculative and states that it was not seen in the record and was not readily available to the Examiner. The Examiner argues that the references relied upon by the Examiner (*e.g.*, the Aicher II article, discussed below) are more recent than any of the articles cited by the Applicants and yet make no assertions of the relationship between the

treatment of Alzheimer's disease and elevated PDH activity.

Claim 18, which has been previously addressed in section (a) and which is believed by Applicants to currently be in condition for allowance, will not be discussed further.

Regarding the enablement of claim 19 directed to the treatment of hyperlipidaemia, Applicants point to, for example, the *New England Journal of Medicine* article by Stacpoole *et al.* ("Stacpoole") that is cited at page 3, lines 9-10 of the specification. A copy of Stacpoole has been attached in furtherance of Applicants' argument. Stacpoole provides the association between the observed hyperlipoproteinemia-lowering (*i.e.*, cholesterol-lowering) properties of compounds (*e.g.*, dichloroacetate) and the compounds' stimulation of pyruvate dehydrogenase (PDH) in peripheral tissues (see, *e.g.*, page 528, 3rd full paragraph and page 529, 5th full paragraph). Although Stacpoole does not propose a mechanism through which PDH activation results in a rapid decrease in plasma lipids, a skilled artisan would nevertheless have a reasonable belief that a PDH-activity-elevating compound would also exhibit these hyperlipidaemia-lowering properties. For at least this reason, claim 19 is enabled and Applicants therefore request that the grounds for rejection of this claim be withdrawn.

Regarding the enablement of claim 20 directed to the treatment of Alzheimer's disease, Applicants point to, for example, the *Journal of Neural Transmission* article by Gibson *et al.* ("Gibson") that is cited at page 3, line 11 of the specification. A copy of Gibson has been attached in furtherance of Applicants' argument. Gibson teaches that neurodegenerative diseases, including Alzheimer's disease, is associated with abnormalities in the pyruvate dehydrogenase complex (PDHC) in the brain (see page 855, Summary). More specifically, Gibson reports that deficiency of PDHC activity has been documented in Alzheimer's disease <u>in at least four independent laboratories</u> (see page 857, 2nd full paragraph). Based on a reading of Gibson, a skilled artisan would have a reasonable belief that a PDH-activity-elevating compound would also be of utility in treating Alzheimer's disease. For at least this reason, claim 20 is enabled and Applicants therefore request that the grounds for rejection of this claim be withdrawn.

The Examiner has provided no support for her allegation that Gibson is a speculative reference. As emphasized above, at least four independent laboratories have come to the

identical conclusion as Gibson regarding the link between PDHC activity and Alzheimer's disease. The apparent lack of disclosure in the documents cited by the Examiner (*e.g.*, Aicher II) of this connection could be due to many factors (*e.g.*, the particular research focus of the authors, selective searching techniques, *etc.*) and should not be considered probative of a lack of such an association.

II. <u>Rejection under 35 U.S.C. § 102(a)</u>

The Examiner maintains her rejection of claims 1, 2, 4-7, 10 and 16-18 under 35 U.S.C. § 102(a) as being anticipated by *Aicher I.* Although the Examiner states that she recognizes that Applicants are claiming benefit under 35 U.S.C. § 119 of a priority application filed September 4, 1999, which predates the publication date of *Aicher I*, the Examiner contends that because Applicants' claims lack enablement under 35 U.S.C. § 112, *Aicher I* is a competent reference.

Applicants repeat their argument that a review of priority documents GB 9920821.7 (filing date: September 4, 1999) and GB 9929835.8 (filing date December 18, 1999), reveal that these documents, when combined, provide full support for at least the subject matter recited in Applicants' claims 1, 2, 4-7, 10 and 16-18. Because Applicants believe that these claims, as amended, are fully enabled by the current specification, which, in turn, is enabled by the priority documents, then Applicants are, at the least, entitled to the latter priority filing date of December 18, 1999. Because this date predates the January 2000 publication date of *Aicher I*, *Aicher I* does not qualify as prior art under 35 U.S.C. § 102(a). Applicants therefore request that this rejection be withdrawn.

III. <u>Conclusion</u>

All remaining grounds for rejection have been fully addressed and overcome by the above amendments and remarks. It is therefore believed that all claims presently pending in this application are allowable. Accordingly, the withdrawal of all grounds for rejection and the allowance of all claims are respectfully requested. The Examiner is invited to contact the undersigned with any questions or concerns that may prevent this requested allowance.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Director is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or to credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3).

Respectfully Submitted,
Morgan Lewis & Bockius LLP

Date: November 15, 2004
Morgan Lewis & Bockius LLP
Customer No. **09629**
1111 Pennsylvania Avenue, N.W.
Washington, D.C. 20004
Tel. No.: 202-739-3000
DJB:GTL

By: ______________________
Donald J. Bird
Registration No. 25,323
Tel. No.: (202) 739-5320
Fax No.: (202) 739-3001

J Neural Transm (1998) 105: 855–870

Journal of Neural Transmission

Abnormalities of mitochondrial enzymes in Alzheimer disease

G. E. Gibson[1,2], **K.-F. R. Sheu**[1,2], and **J. P. Blass**[1,2,3]

[1] Burke Medical Research Institute, White Plains, NY, and
[2] Department of Neurology and Neuroscience, and
[3] Department of Medicine, Cornell University Medical College, New York, NY, USA

Received April 29, 1998; accepted May 20, 1998

Summary. Abundant evidence, including critical information gathered by Prof. Siegfried Hoyer and his colleagues, indicates that abnormalities of cerebral metabolism are common in neurodegenerative diseases, including Alzheimer's Disease (AD). Alterations in mitochondrial enzymes likely underlie these deficits. Replicable reductions in AD brain occur in the pyruvate dehydrogenase complex (the link of glycolysis to the Kreb's cycle), the α-ketoglutarate dehydrogenase complex (KGDHC; the link of Kreb's cycle to glutamate metabolism) and cytochrome oxidase (the link of the Kreb's cycle to oxygen utilization). Available evidence suggests that deficiencies in KGDHC may be genetic in some cases, whereas evidence that the other two enzyme systems have a genetic component is lacking. Additional results indicate that the reductions can also be secondary to other causes including oxidative stress. A variety of data suggest that the mitochondrial insufficiencies contribute significantly to the pathophysiology of AD.

Keywords: Alzheimer's disease, mitochondria, α-ketoglutarate dehydrogenase, cytochrome oxidase, pyruvate dehydrogenase, neurodegenerative disease.

Introduction

Extensive evidence gathered over the last 50 years and reviewed elsewhere (Blass, 1993a,b, 1997) indicates that the rate of cerebral metabolism is reduced in Alzheimer's Disease (AD). Decreased cerebral metabolism precedes the development of clinical (neuropsychological) or neuroanatomic (imaging) evidence of the disease. These data were gathered by PET scanning of subjects whose familial and/or APOE status put them at very high risk to develop AD (Reiman et al., 1996; Small et al., 1995). Hoyer and colleagues (Baur et al., 1997; Blum-Degen et al., 1995; Hoyer, 1996, 1997) have shown that alterations in the pattern of substrates metabolized by brain accompany the very earliest clinical signs of AD (Hoyer, 1996, 1997). These deficits in brain

metabolism are likely to be important to diminished brain function since treatment with glucose causes a statistically significant improvement in mentation, as measured by neuropsychological testing, in patients with AD but not in non-AD controls (Craft et al., 1992; Manning et al., 1993). Four lines of evidence suggest that alterations in mitochondrial enzymes underlie these deficits:

- Measurements of mitochondrial enzyme activities indicate inherent damage to mitochondria in AD brain (Perry et al., 1980; Sorbi et al., 1983; Yates et al., 1990; Butterworth and Besnard, 1990; Gibson et al., 1988; Mastrogiacomo et al., 1994; Chandrasekaran et al., 1996; Kish et al., 1992; Simonian and Hyman, 1994). The mitochondrial enzyme complexes for which this point has been studied in detail include the α-ketoglutarate dehydrogenase complex (KGDHC), the pyruvate dehydrogenase complex (PDHC), and cytochrome oxidase (COX).
- Two approaches indicate that genetic abnormalities in these enzymes may underlie their reduction in Alzheimer brain. Molecular genetic studies document an association between AD and a gene, DLST, which encodes a critical component of the KGDHC complex (Ali et al., 1994; Sheu et al., 1996, 1997, 1998a; Nakano et al., 1994, 1997). Changes in COX persist following transfer of mitochondrial DNA from Alzheimer patient platelets to neuroblastoma cells depleted of endogenous mtDNA (Swerdlow et al., 1997), although this is not necessarily evidence of changes in the mitochondrial CO1 and CO2 genes encoding components of COX.
- These enzymes can also change secondarily to other pathologic events in AD, including oxidative stress. Evidence suggests that this secondary reduction may be part of a critical cascade of events that lead to neurodegeneration.
- Interference with these enzymes can be readily related to the pathophysiology of AD.

1. Activities of specific mitochondrial enzyme complexes are reduced in AD

Deficient activities of three mitochondrial enzyme complexes have been reported in AD: cytochrome oxidase (COX), the pyruvate dehydrogenase complex (PDHC), and the α-ketoglutarate dehydrogenase complex (KGDHC). Normal activities have been found for several other mitochondrial enzymes, including fumarase [a component of the Krebs tricarboxylic acid cycle (Sorbi et al., 1983)], glutamate dehydrogenase [an enzyme coupling glutamate to KGDHC (Gibson et al., 1988)] and components of the electron transport chain such as COX (Kish et al., 1992; Simonian and Hyman, 1994).

COX is complex IV of the mitochondrial electron transport chain, the component of the electron transport chain which interacts directly with molecular oxygen. Kish et al. (1992) that COX activity declined moderately in cerebral cortex of 29 AD patients compared to 29 control subjects. COX in cerebral cortex was reduced by 16–26% in the AD group. COX activity in AD has been reported to be modestly reduced in 9 independent studies ($p < 0.05$ in

5), since the original report (see Kish, 1997 for a detailed discussion). Deficiency of COX has been described in both severely and less severely histologically affected areas of brain (Chandrasekaran et al., 1996; Kish et al., 1992; Simonian and Hyman, 1994). The extent and region of the reductions vary among reports. For example, some (Wong-Riley et al., 1997), but not other (Simonian and Hyman, 1994) studies find differences in visual cortex. The pattern of down-regulation for both mitochondrial and nuclear gene expression coding for subunits of COX in the AD brain resembles those in normal brain caused by chronic sensory deprivation (Chandrasekaran et al., 1996). Thus, the results suggest a generalized suppression of oxidative metabolism throughout the cortex (Wong-Riley et al., 1997) and appear to reflect physiological down regulation of COX gene expression (Chandrasekaran et al., 1996). Physiological down-regulation of COX gene expression in AD is consistent with PET evidence that cognitive or psychophysical activation of mildly to moderately demented Alzheimer's patients can augment brain-blood flow and glucose metabolism to the same extent as in control subjects (Chandrasekaran et al., 1996). Evidence that oxidative metabolism can be rapidly activated is also provided by studies showing that glucose causes significant improvement in mentation in AD (Craft et al., 1992; Manning et al., 1993). Nevertheless, some reports suggest that COX structure may be modified in AD; COX purified from AD brain showed a loss of one of the two kinetically identifiable sites for reduced cytochrome c (Parker and Parks, 1995).

Persistence of AD-related deficits in non-neuronal tissues suggest that the abnormalities are not just secondary to neurodegeneration, and that they reflect constitutive properties of the cells. Deficiency of COX activity in platelets has been reported (Parker et al., 1990) and disputed (VanZuylen et al., 1992). Fibroblasts from AD patients display decreased COX activity ($P < 0.05$; Curti et al., 1997), whereas lymphocytes do not have altered activities (Molina et al., 1997).

PDHC catalyzes the reaction by which pyruvate (usually derived from glucose) is converted to acetyl-CoA which then enters the Krebs tricarboxylic acid cycle (Sorbi et al., 1983). This large multi-enzyme complex is composed of three major and two minor protein components (Sorbi et al., 1983). Deficiency of PDHC activity has been documented in AD brain, in at least four independent laboratories (Sorbi et al., 1983; Perry et al., 1980; Yates et al., 1990; Butterworth and Besnard, 1990) with no contravening reports. The enzyme is deficient in both histologically affected and histologically unaffected areas of brain (Sorbi et al., 1983). However, repeated studies under a number of different experimental conditions have not shown reliable deficiencies in PDHC in non-neural tissues, and specifically none in cultured AD skin fibroblasts (Blass, unpublished results). Nor have preliminary studies demonstrated any evidence for an association between AD and genes encoding components of the PDHC complex (Blass, unpublished results). Thus, available evidence does not argue for a primary genetic abnormality in PDHC in an appreciable proportion of patients with AD.

KGDHC catalyzes a critical reaction within the Krebs tricarboxylic acid cycle, namely the oxidation of α-ketoglutarate to succinyl-CoA (Blass,

1993a,b, 1997; Gibson et al., 1988). KGDHC is also an important enzyme in glutamate metabolism, since α-ketoglutarate is readily interconverted with glutamic acid by transamination and is the product of glutamate oxidation by the glutamate dehydrogenase catalyzed reaction (Blass, 1997). KGDHC, like PDHC, is a large multi-enzyme complex with three major protein components. In KGDHC, these are E1k, E2k, and E3. E1k is α-ketoglutarate dehydrogenase, which is encoded on the OGDH gene on chromosome 7p13–p11 (Szabo et al., 1994). E2k is dihydrolipoyl succinyltransferase, and is encoded on the DLST gene on chromosome 14q24.3 (Ali et al., 1994; Nakano et al., 1994). E3 is dihydrolipoamide dehydrogenase and is a common component of the PDHC and KGDHC dehydrogenase complexes. It is encoded by the DLD gene on chromosome 7q31–q32 (Scherer et al., 1991).

KGDHC activity is reduced in AD brain, in both histopathologically affected and histopathologically unaffected areas of brain (Gibson et al., 1988; Mastrogiacomo et al., 1994; Butterworth and Besnard, 1990). This finding, originally reported by our group at Burke (Gibson et al., 1988), is robust. It has been replicated independently in at least two other laboratories (Mastrogiacomo et al., 1994; Butterworth and Besnard, 1990) as well as in several independent studies in this laboratory; there are no contravening reports. To determine whether the reduction in brain KGDHC activity in AD is associated with an abnormality in one of its three constituent enzyme subunits, Kish and coworkers measured the protein levels of E1k, E2k and E3 in postmortem brains of 29 patients with AD (mean age, 73 years; age range of onset, 50–78 years) and 29 control subjects (Mastrogiacomo et al., 1996). In the AD group protein levels of all three subunits were significantly reduced by 23 to 41% in the temporal cortex, whereas in the parietal cortex (E1: −28%; E3: −32%) and hippocampus (E3: −33%) significant changes were limited to E1k and E3. KGDHC activities were more markedly reduced (by 46–68%) and did not correlate with protein levels, suggesting that decreased enzyme activity cannot be directly explained by loss of KGDHC protein.

Activity of KGDHC has also been found to be reduced in cultured skin fibroblasts from "sporadic" patients with AD (Blass et al., 1997a,b) and in some (Sheu et al., 1994) but not all (Blass et al., 1997a) patients with presenilin-1 mutations. Two groups have found reduced oxidation of [$U^{14}C$]glutamine to $^{14}CO_2$ in AD compared to non-AD control cultured skin fibroblasts (Sims et al., 1987; Peterson and Goldman, 1986). The reduced oxidation of glutamine in AD cells is also consistent with a functional block in KGDHC, since glutamine rapidly converts to glutamate which is readily converted to α-ketoglutarate by either transamination or the glutamate dehydrogenase catalyzed reaction (Blass, 1993a,b, 1997). However, the oxidation of [^{14}C]-ketoglutarate does not vary between control and AD cells, nor does amino acid metabolism as measured by the incorporation of $^{13}NH_3$ into relevant amino acids, in cultured AD fibroblasts (Cooper et al., 1996).

Thus, the data that reductions in the activities of these key enzymes occur in AD brain occur and that such decreases could underlie the reduced metabolism in vivo are strong and replicable. Superficially, studies of biopsy brain from living patients are difficult to reconcile with this data. For instance,

pyruvate/malate-dependent oxygen consumption, measured directly with an O_2 electrode in homogenates of freshly biopsied frontal neocortex in AD and non-AD human brain under carefully standardized conditions, is not decreased either with or without ADP or in the presence of CCCP, which uncouples oxidation from phosphorylation (ATP production) (Sims et al., 1987b). These results indicate that rates of PDHC and oxygen utilization by COX can be normal in AD brain, at least under the saturating conditions used in these experiments. In earlier studies, the rate of conversion of [U-^{14}C]glucose by slices of biopsied AD brain was 39% higher compared to controls (Sims et al., 1981, 1983). This result is consistent with a lack of functional control on pyruvate oxidation, as suggested by the studies of the human brain homogenates (Sims et al., 1987a,b). Thus, the biopsy studies suggest that neither PDHC nor COX are limiting in AD brain.

The apparent contradiction between reduced activities of PDHC and COX in AD brain at autopsy but normal or increased oxidation of pyruvate or glucose in the ex vivo experiments suggests that different phenomena are being measured in the two types of studies. The measurements in autopsy brain were of total enzymatic activity under conditions where mitochondria had been disrupted and mitochondrial control mechanisms were therefore lost. In those studies, the deficiency in PDHC activity in AD brain appeared to be associated with a reduced amount of immunochemically normal enzyme complex (Sheu et al., 1985). The majority of the data on the deficiency of COX suggests that it is associated with down-regulation of this complex (see above). The measurements of enzyme activity are therefore compatible with a reduction (i) in the number of mitochondria, (ii) in the amount of PDHC and/or COX per mitochondrion, or (iii) with a reduction in PDHC and COX activity in a subgroup of damaged mitochondria (e.g., from damaged cells). The normal values for maximal (CCCP-stimulated) O_2 uptake in AD brain in the ex vivo studies argue against a dramatic reduction in the number of mitochondria in these patient samples, although other explanations are possible. Possibly, oxygen uptake measured under the conditions of these experiments was carried out by mitochondria which were largely intact in AD brain. The mitochondria in the human brain microslices derive essentially from synaptosomes (Sims et al., 1981), and different populations of brain mitochondria can have strikingly different compositions (Westergaard et al., 1995), creating an experimental difference between the autopsy and biopsy studies. Another possibility is that in the intact AD mitochondria in the homogenates studied in the ex vivo experiments, the rate of oxidation was limited not by the PDHC or COX activities but by as yet unspecified control mechanisms or perhaps by later steps in the Krebs cycle, such as that catalyzed by KGDHC (Blass, 1993a,b). Further experiments to explore these issues have been hindered by a lack of biopsy material from AD and control patients.

The quantitative relationships among these enzymes in normal human frontal cortex and in AD are shown in Fig. 1. Choline acetyltransferase, the synthetic enzyme for acetylcholine which is known to be diminished in AD brain, is shown as a reference. Figure 1A shows that the activities of the dehydrogenase complexes are an order of magnitude or more lower than

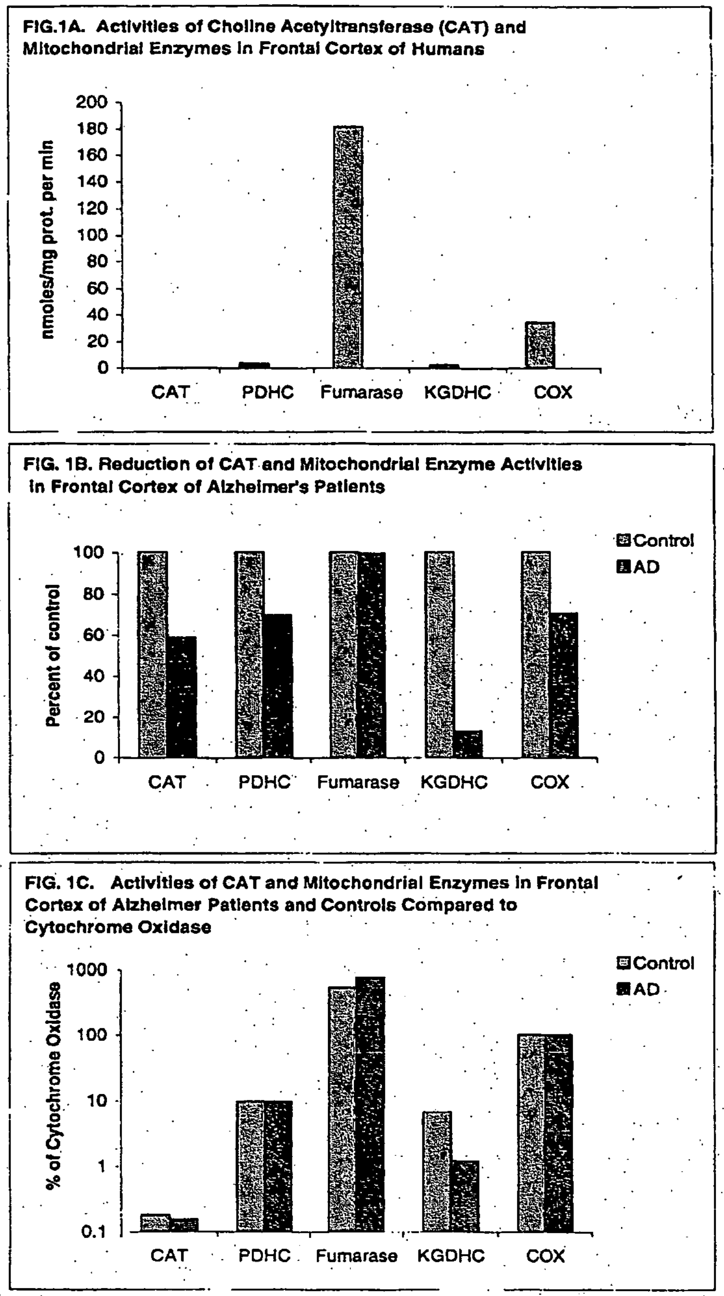


Fig. 1. Enzyme activities in Alzheimer's Disease brain. In **A**, the relative enzyme values (nmoles/mg proteins per minute) are shown for CAT (0.063; Kish et al., 1992), PDHC (3.3; Sorbi et al., 1982), fumarase (Kish et al., 1992), KGDHC (2.25; Gibson et al., 1988), COX (34; Kish et al., 1992). The calculated values for the derived values in **B** and **C** are from these same references

those of COX or fumarase, with KGDHC being slightly lower than PDHC (Fig. 1A). The decline in activities with AD in KGDHC is more severe than for the other mitochondrial enzymes or choline acetyltransferase (Fig. 1B). As shown in Fig. 1C, KGDHC but not PDHC or fumarase showed reduced activities compared to the COX activity (remaining) in AD brain. There are risks in trying to extrapolate enzyme activities measured under forcing conditions to enzyme activities within biopsies to the control mechanisms operating in living cells. Nevertheless, the data shown in the figure are consistent with KGDHC being a potentially rate-limiting enzyme (i.e., having a relatively high control coefficient) in cerebral oxidative metabolism.

2. Genetic abnormalities may underlie enzymatic abnormalities in mitochondrial enzymes in AD brain

COX

Studies of cybrids have been interpreted to implicate genetic abnormalities of mtDNA COX genes in AD (Swerdlow et al., 1997). Cybrids are cells which have been depleted of their own mtDNA, which are then repopulated with mitochondria from AD or control subjects. AD cybrids have reduced COX, changes in calcium regulation and elevated free radical production (Sheehan et al., 1997). These studies are relatively new and independent replication of the experiments with cybrids has not been reported. A recent report proposed the existence in AD of discrete mutations in mitochondrial DNA (mtDNA) of the CO1 and CO2 genes encoding the corresponding components of COX (Davis et al., 1997), but subsequent studies (Wallace et al., 1997; Hirano et al., 1997) indicated that the apparent "mutations" were attributable to PCR coamplification of pseudogenes for CO1 and CO2 on nuclear DNA (nDNA). While the reported alteration in nDNA pseudogene/mtDNA gene amplification suggests that there is, indeed, some difference between mtDNA in AD and control tissues, the current evidence argues against the existence of discrete AD-associated mutations in the CO1 or CO2 genes of mtDNA (Wallace et al., 1997; Hirano et al., 1997). In AD brain, a significant decrease was found in the ratio of PCR amplification of the mtDNA CO1 gene when compared to the nuclear DNA (nDNA) CO1 pseudogene when compared to age and sex matched non-AD controls (Brown et al., 1998). However, the study provided no evidence for a mutation of CO1 gene in AD. Nor is there any evidence of abnormalities in nDNA genes encoding components of COX. Several reports have suggested that point mutations of the mitochondrial DNA (mtDNA) might contribute to the pathogenesis of AD. A recent study screened brain tissue from 65 AD patients for each of the previously reported mtDNA mutations. The results failed to demonstrate an increased incidence of any of the mutations in the AD cases (Hutchin et al., 1997). Thus, the role of mtDNA mutations in the pathogenesis of AD remains unclear.

KGDHC

AD is associated with polymorphisms of the DLST gene, which encodes a component of KGDHC. Molecular genetic studies in our unit have indicated

that polymorphisms of DLST, which encode the core protein of KGDHC, are associated with AD (Sheu et al., 1994, 1996, 1997, 1998a,b). Association of AD with polymorphisms of DLST have now been found in over 1,400 subjects, studied in three different laboratories on three different continents (Sheu et al., 1996, 1998; Nakano et al., 1997). Associations have been observed in both familial (APP- and PS1-negative) AD and apparently sporadic AD (Sheu et al., 1998a,b; Lilius et al., in preparation), and in patients who carry the ϵ-4 allele of the APOE gene (APOE4 positive) and in subjects who are APOE4 negative (Sheu et al., 1998). Thus, a genetic association with AD at this locus is a confirmed finding.

The polymorphisms of DLST which have been associated with AD are A19117G in intron 13 and T19183C in exon 14. Homozygosity for the G,C allele has been shown to enhance the association of APOE4 with AD in our Caucasian Jewish series of 429 subjects, and homozygosity for A,C/A,C with AD in the Japanese series of 251 patients and 452 controls (Nakano et al., 1997). The T19183C mutation does not alter the amino acid composition of the DLST protein; both bases encode a glycine (codon 366). It is hard to postulate how this silent polymorphism would lead to a pathophysiologically important change. The intron 13 polymorphism is a base change at a potential branch site, and therefore might conceivably affect mRNA processing. However, it is hard to visualize a mechanism by which A at position 19,117 would be pathogenetic in Japanese and G in Caucasians.

The hypothesis we currently favor is that the polymorphisms of DLST so far discovered to be associated with AD are markers for other, not yet elucidated pathogenetic alterations linked to these markers. The molecular genetic data by themselves are compatible with a defect in either DLST or in a nearby gene on chromosome 14q24.3. However, the combination of biochemical and genetic data make it likely that the genetic abnormality is in the DLST gene itself.

3. Mitochondrial enzymes can also be affected secondarily by other mechanisms in AD, including oxidative stress

In one of the few AD families in which the causative gene defect is known (i.e., the APP670/671 mutation that leads to overproduction of amyloid-β-peptide), KGDHC is also down in brain. In patients bearing the APP670/671 mutation, KGDHC activities were reduced 55–57% compared to control values. The immunochemical levels of KGDHC subunits E1k (−51%), E2k (−76%) declined, while E3 concentrations were unchanged. The results with the APP670/671 mutation bearing patients suggest that mitochondrial dysfunction is a part of the pathophysiological process in AD even when the primary pathogenic event is non-mitochondrial (Gibson et al., 1997).

The evidence that oxidative stress is involved in the pathophysiology of AD is very strong (Markesbery, 1997). However, the mechanisms leading to this manifestation of the disease are as yet undetermined. The toxic effects of Alzheimer β-amyloid appear to be mediated by oxidative stress (Markesbery, 1997), and the "toxic fragment" of the Aβ peptide appears to form spontane-

ously free radicals which are not formed by the scrambled or reverse peptides (Yatin et al., 1997). Furthermore, the toxic effects of Aβ are ameliorated by the addition of a variety of free radical scavengers (Bruce et al., 1996; Tomiyama et al., 1996; Richardson et al., 1996; Kumar et al., 1994; Goodman et al., 1994; Goodman and Mattson, 1994). Both inflammation and apoptosis are associated with free radical mechanisms, and both these processes occur in AD (Markesbery, 1997; Blass, 1996).

Oxidative stress can damage COX, KGDHC and PDHC, although these interactions have not been studied in detail in brain. Depletion of glutathione levels in cultured cardiac myocytes significantly reduced pyruvate dehydrogenase activity (Tirmenstein et al., 1997). A study on the relative susceptibility of (membrane-associated, contractile and mitochondrial) proteins in normal human muscle to oxidative damage by ROS revealed that succinate dehydrogenase (complex II) and cytochrome oxidase (complex IV) were particularly susceptible. At the ultrastructural level, mitochondria were identified as being particularly susceptible to ROS induced oxidative damage. Thus, oxidative damage to mitochondria and/or mitochondrial proteins may represent the principal initial route of free radical-induced damage within skeletal muscle tissue (Haycock et al., 1996), and a similar process may occur in neurodegenerative diseases.

The effects of oxidative stress on KGDHC have been studied more comprehensively. The sulfhydryl groups of the FAD containing E3 may make KGDHC particularly vulnerable to free radicals. Cellular intoxication by elevated concentrations of O_2 results in excessive free radical production by normal metabolic pathways. Exposure of cultures of either HeLa cells or Chinese hamster ovary (CHO) cells to 80% O_2 for 2 days causes progressive growth inhibition and loss of reproductive capacity. This impairment is correlated with inhibition of cellular O_2 consumption and partial inactivation of NADH and succinate dehydrogenase, and total inactivation of KGDHC (Schoonen et al, 1990a). In an oxygen-resistant substrain of CHO cells, succinate dehydrogenase and KGDHC are relatively resistant to inactivation by hyperoxia (Shoonen et al., 1991, 1990b). This finding supports the suggestion that damage to these enzymes is a critical part of a cascade in the pathological response to free radicals in non-mutant CHO cells.

Mitochondrial damage may also be predicted to lead to oxidative stress by at least two mechanisms. One is increased production of ROS due to damage to the electron transport chain; for instance, loss of activity of COX might back up electrons at center P of the Q cycle portion of the Complex III site with the possible formation of ROS (Kristal et al., 1997). The other mechanism is impaired ROS sequestration due to impaired chemical flux. Removal of ROS eventually requires their chemical reduction, and a functional deficiency in the Krebs tricarboxylic acid cycle can be predicted to lead to reduced production of the electrons (i.e., NADH equivalents) which are needed for the chemical reduction of ROS. A functional deficiency of KGDHC could be predicted to favor increased ROS production (Blass 1993a, 1996, 1997). Free radical/ROS mechanisms in relation to AD are now under study in many laboratories (Markesbery, 1997).

Nitric Oxide (NO) production in the brain can also lead to oxidative stress (Bolanos et al., 1997). Under certain circumstances NO synthesis may be excessive and NO may become neurotoxic. This is particularly true in the presence of superoxide, since the combination of superoxide and NO forms peroxynitrite. Peroxynitrite can interfere with key enzymes of the tricarboxylic acid cycle, the mitochondrial respiratory chain, or mitochondrial calcium metabolism, and/or cause DNA damage with subsequent activation of the energy-consuming pathway involving poly(ADP-ribose) synthetase (Bolanos et al., 1997). Recent results indicate that oxidative stress in the presence of NO leads to peroxynitrite formation, which leads to the nitration and inactivation of KGDHC (Park et al., 1998).

4. Impaired mitochondrial metabolism can be readily linked to the pathophysiology of Alzheimer's disease

KGDHC, PDHC and COX may be critical steps in the cascade of events that lead to AD, regardless of whether they are primary genetic defects or secondary events due to oxidative stress. PDHC activity has been linked to key aspects of major neuropathologic correlates of AD (i.e., the plaques, tangles and cholinergic deficit). PDHC can be phosphorylated and inactivated in vivo by tau protein kinase (TPKI), a kinase that is critical to the formation of tangles in AD brain (Imahori and Uchida, 1997), reduces PDHC activity (Imahori and Uchida, 1997). In primary culture of rat hippocampal cells, amyloid-β-peptide, the main component of plaques, inactivated PDH in inverse relation to its effects on the activation of TPKI. The amyloid-β-peptide-induced inhibition of PDHC lead to accumulation of pyruvate or lactate, energy failure, and a shortage of acetylcholine, all of which are characteristic of AD brain. Neither choline acetyltransferase activity nor choline metabolism is affected by the kinase. Therefore, the major cause of reduced acetylcholine synthesis is likely to be an inadequate supply of acetyl-CoA due to reduced PDHC activity.

Relatively extensive studies of the biochemical pathophysiology of AD have documented a variety of mechanisms other than oxidative stress which can relate a deficiency in mitochondrial substrate oxidation (i.e. in energy/oxidative metabolism) to specific clinical and pathological characteristics of AD. These have been reviewed elsewhere (Blass, 1993a,b, 1996, 1997). They include cognitive impairments, neurotransmitter and specifically cholinergic impairments, abnormalities in signal transduction similar to those seen in AD, cytoskeletal lesions, and abnormal processing of amyloid precursor protein (Blass, 1993a,b, 1996, 1997). Thus there is a body of work supporting the pathophysiological significance of mitochondrial abnormalities in AD.

5. Implications

A body of pathophysiological, biochemical, and molecular genetic studies all support the importance of studying the mitochondrial lesion in AD. Mitochondrial abnormalities appear to be a common mechanism in AD from a variety of genetic causes. Accumulating evidence indicates that a mutation of

a gene encoding a mitochondrial constituent may predispose to the development of the disease in a proportion of AD patients. In other patients, genetic abnormalities in other genes including APP and the presenilins appear to lead to mitochondrial abnormality (Cruts et al., 1998; Rubinsztein, 1997; Farrer et al., 1997), perhaps via free radical formation (Markesbery, 1997).

The existence of primary genetic and secondary abnormalities in a mitochondrial component in AD are, of course, not mutually exclusive. For instance, there is no contradiction between evidence that deficiency of KGDHC in AD can sometimes be associated with polymorphisms in a gene encoding the core component of KGDHC and at other times with inactivation of KGDHC by peroxynitrites or other reactive oxygen species (ROS). Conceivably, the effect of the genetic abnormality may be to make the encoded protein more sensitive to damage by ROS. Diseases with multiple genetic causes may be more common than single gene diseases, and the common forms of AD are being increasingly accepted to be polygenetic disorders. Possibly, a gene encoding a specific component of the mitochondrion may interact with the effects of other genes encoding other components (non-mitochondrial or mitochondrial) in leading to the impairments which are the pathophysiological mechanisms of AD. Current evidence for this possibility is strongest for KGDHC and for the DLST gene which encodes its core protein component. These potential interactions are diagrammed in Fig. 2.

Mitochondrial insufficiency can be related to the pathophysiology of AD by plausible and well-established mechanisms for which there is abundant evidence. Further studies of the mitochondrial deficit in AD are warranted.

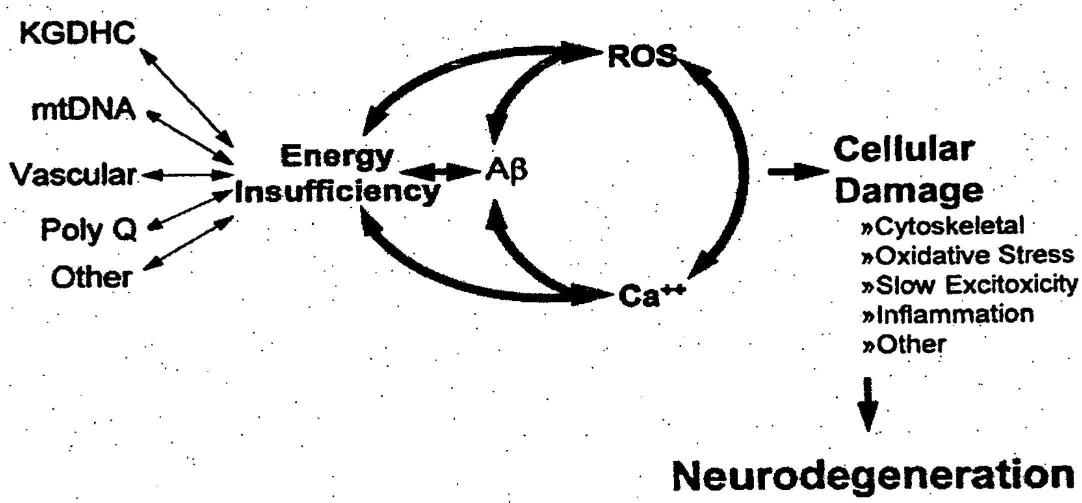


Fig. 2. Pathophysiological mechanisms in Alzheimer's disease (from Blass et al., 1997b)

Acknowledgements

Supported by NIH grants AG14600 (GEG) and AG11921 (GEG), AG09014 (JPB) and AG14930 (JPB) and by funds from the Overbrook Foundation and the Winifred Masterson Burke Foundations.

References

Ali G, Wasco W, Cai X, Szabo P, Sheu K-FR, Cooper AJ, Gaston SM, Gusella JF, Tanzi R, Blass JP (1994) Isolation, cloning, and localization of the gene for the E2k component of the human α-ketoglutarate dehydrogenase complex. Somat Cell Mol Genet 20: 99–105

Bauer J, Plaschke K, Martin E, Bardenheuer HJ, Hoyer S (1997) Causes and consequences of neuronal energy deficit in sporadic Alzheimer's disease. Ann NY Acad Sci 826: 379–381

Blass JP (1993a) Pathophysiology of the Alzheimer syndrome. Neurol 43 [Suppl 4]: S25–S38

Blass JP (1993b) Metabolic alterations common to neural and non-neural cells in Alzheimer's disease. Hippocampus 3: 45–54

Blass JP (1996) Alzheimer's disease: melting pot or mosaic? Alz Dis Rev 1: 1720

Blass JP (1997) Cerebral metabolic impairments. In: Alzheimer's disease: cause(s), diagnosis, treatment, and care. CRC Press, New York, pp 187–206

Blass JP, Sheu K-FR, Piacentini S, Sorbi (1997a) Inherent abnormalities in oxidative metabolism in AD: interaction with vascular abnormalities. Ann NY Acad Sci 826: 382–385

Blass JP, Gibson GE, Hoyer SE (1997b) Metabolism of the aging brain. Adv Cell Aging Gerontol 2: 109–128

Blum-Degen D, Frölich L, Hoyer S, Riederer P (1995) Altered regulation of brain glucose metabolism as a cause of neurodegenerative disorders? J Neural Transm [Suppl] 46: 139–147

Bolanos JP, Almeida A, Stewart V, Peuchen S, Land JM, Clark JB, Heales SJ (1997) Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases. J Neurochem 68: 2227–2240

Brown AM, Sheu K-FR, Haroutunian V, Mohs R, Blass JP (1998) Abnormalities of mitochondrial CO1 gene in Alzheimer's disease brain. J Neurochem 70 [Suppl]: S48A

Bruce AJ, Malfroy B, Baudry M (1996) beta-Amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic catalytic free radical scavenger. Proc Natl Acad Sci (USA) 93: 2312–2316

Butterworth RF, Besnard AM (1990) Thiamine-dependent enzyme changes in temporal cortex of patients with Alzheimer's disease. Metab Brain Dis 5: 179–184

Chandrasekaran K, Hatanpaa K, Brady DR, Rapoport SI (1996) Evidence for physiological down-regulation of brain oxidative phosphorylation in Alzheimers disease. Exp Neurol 142: 80–88

Cooper AJL, Sheu K-FR, Blass JP (1996) Normal glutamate metabolism in Alzheimer's disease fibroblasts deficient in α-ketoglutarate dehydrogenase activity. Dev Neurosci 18: 499–504

Craft S, Zallen G, Baker LD (1992) Glucose and memory in milde senile dementia of the Alzheimer type. J Clin Exp Neuropsych 14: 253–267

Cruts M, van Dujin CM, Backhovens H, Van den Broeck M, Wehnert A, Serneels S, Sherrington R, Hutton M, Hardy J, St George-Hyslop PH, Hofm A, Van Broeckhoven C (1998) Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease. Hum Mol Genet 7: 43–51

Curti D, Rognoni F, Gasparini L, Cattaneo A, Paolillo M, Racchi M, Zani L, Bianchetti A, Trabucchi M, Bergamaschi S, Govoni S (1997) Oxidative metabolism in cultured

fibroblasts derived from sporadic Alzheimer's disease (AD) patients. Neurosci Lett 236: 13–16

Davis RE, Herrnstadt C, Ghosh SS, Fahey E, Shinobu LA, Balasko D, Thal LJ, Beal MF, Howell N, Parker WD (1997) Mutations in mitochondrial cytochrome C oxidase genes segregate with late onset Alzheimer's disease. Proc Natl Acad Sci USA 94: 4526–4531

Farrer LA, Adrienne-Cupples L, Haines JL, Hyman B, Kukull WA, Mayeux R, Mayers RH, Pericek-Vance MA, Risch N, van Dujin CM, for the APOE and Alzheimer Disease Meta Analysis Consortium (1997) Effects of age, sex, and ethnicity on the association between Apolipoprotein E genotype and Alzheimer's disease. A meta-analysis. J Am Med Assoc 278: 1349–1356

Gibson GE, Sheu K-FR, Blass JP, Baker A, Carlson KC, Harding B, Perrino P (1988) Reduced activities of thiamine-dependent enzymes in the brains and peripheral tissues of patients with Alzheimer's disease. Arch Neurol 45: 836–840

Gibson GE, Zhang H, Sheu KF-R, Bogdanovich N, Lannfelt L, Vestling M, Cowburn RF (1997) Oxidative enzymes in Alzheimer's disease neurodegeneration. Soc Neurosci 23: 637.5

Goodman Y, Mattson MP (1994) Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. Exp Neurol 128: 1–12

Goodman Y, Steiner MR, Steiner SM, Mattson MP (1994) Nordihydroguaiaretic acid protects hippocampal neurons against amyloid beta-peptide toxicity, and attenuates free radical and calcium accumulation. Brain Res 654: 171–176

Haycock JW, Jones P, Harris JB, Mantle D (1996) Differential susceptibility of human skeletal muscle proteins to free radical induced oxidative damage: a histochemical, immunocytochemical and electron microscopical study in vitro. Acta Neuropathol 92: 331–340

Hirano M, Shtilbans A, Mayeux R, Davidson MM, DiMauro S, Knowles JA, Schon EA (1997) Apparent mtDNA heteroplasmy in Alzheimer's disease patients and in normals due to PCR amplification of nucleus-embedded mtDNA pseudogenes. Proc Natl Acad Sci USA 94: 14894–14899

Hoyer S (1996) Oxidative metabolism deficiencies in brains of patients with Alzheimer's disease. Acta Neurol Scand [Suppl] 165: 18–24

Hoyer S (1997) Models of Alzheimer's disease: cellular and molecular aspects. J Neural Transm [Suppl] 49: 11–21

Hutchin TP, Heath PR, Pearson RCA, Sinclair AJ (1997) Mitochondrial DNA mutations in Alzheimer's disease. Biochem Biophys Res Comm 241: 221–225

Imahori K, Uchida T (1997) Physiology and pathology of tau protein kinases in relation to Alzheimer's disease. J Biochem 121: 179–88

Kish SJ (1997) Brain energy metabolizing enzymes in Alzheimer's disease: alpha-ketoglutarate dehydrogenase complex and cytochrome oxidase. Ann NY Acad Sci 826: 218–228

Kish SJ, Bergeron C, Rajput A, Dozic S, Mastrogiacomo F, Chang F, Wilson JM, DiStefano LM, Nobrega JN (1992) Brain cytochrome oxidase in Alzheimer's disease. J Neurochem 59: 776–779

Kristal BS, Jackson CT, Chung HY, Matsuda M, Nguyen HD, Yu BP (1997) Defects at center P underlie diabetes-associated mitochondrial dysfunction. Free Rad Biol Med 22: 823–833

Kumar U, Dunlop DM, Richardson JS (1994) The acute neurotoxic effect of beta-amyloid on mature cultures of rat hippocampal neurons is attenuated by the anti-oxidant U-78517F. Int J Neurosci 79: 185–190

Manning CA, Ragozzino ME, Gold PE (1993) Glucose enhancement of memory in patients with probable dementia of the Alzheimer's type. Neurobiol Aging 14: 523–528

Markesbery WR (1997) Oxidative stress hypothesis in Alzheimer's disease. Free Radic Biol Med 23: 134–147
Mastrogiacomo F, Bergeron C, Kish SJ (1994) Brain α-ketoglutarate dehydrogenase activity in Alzheimer's disease. J Neurochem 61: 2007–2014
Mastrogiacomo F, Lindsay JG, Bettendorf L, Rice J, Kish SJ (1996) Brain protein and α-ketoglutarate dehydrogenase complex activity in Alzheimer's disease brain. Ann Neurol 39: 592–598
Molina JA, Debustos F, Jimenezjimenez FJ, Benitoleon J, Gasalla T, Ortipareja M, Vela L, Bermejo F, Martin MA, Campos Y, Arenas J (1997) Respiratory chain enzyme activities in isolated mitochondria of lymphocytes from patients with Alzheimer's disease. Neurol 48: 636–638
Nakano K, Takase C, Sakamoto T, Nakagawa S, Inazawa J, Ohta S, Matuda S (1994) Isolation, characterization, and structural organization of the gene and pseudogene for the dihydrolipoyl succinyltransferase component of the human 2-oxoglutarate dehydrogenase complex. Eur J Biochem 224: 179–189
Nakano K, Ohta S, Nishimaki K, Miki K, Matsuda S (1997) Alzheimer's disease and DLST genotype. Lancet 350: 1367–1368
Park CH, Calingasan NY, Sheu KF-R, Kristal BS, Zhang H, Gibson GE (1998) Oxidative stress compromises inflammatory responses and a key mitochondrial enzyme in microglia. J Neurochem 70S: S57B
Parker WD, Parks JK (1995) Cytochrome c oxidase in Alzheimer's disease brain: purification and characterization. Neurol 45: 482–486
Parker WD, Filley CM, Parks JK (1990) Cytochrome oxidase deficiency in Alzheimer's disease. Neurol 42: 1246–1247
Perry EK, Perry RH, Tomlinson G, Blessed G, Gibson PH (1980) Coenzyme-A acetylating enzymes in Alzheimer disease: possible cholinergic "compartment" of pyruvate dehydrogenase. Neurosci Lett 18: 105–110
Peterson C, Goldman JE (1986) Alterations in calcium content and biochemical processes in cultured skin fibroblasts from aged and Alzheimer donors. Proc Natl Acad Sci USA 83: 2758–2762
Reiman EM, Caselli RJ, Yun LS, Chen K, Bandy D, Minoshima S, Thibodeau SN, Osborne D (1996) Preclinical evidence of Alzheimer's disease in persons homozygous for the ∊4 allele for apolipoprotein E. N Engl J Med 33: 752–758
Richardson JS, Zhou Y, Kumar U (1996) Free radicals in the neurotoxic actions of beta-amyloid. Ann NY Acad Sci 777: 362–367
Rubinsztein DC (1997) The genetics of Alzheimer's disease. Prog Neurobiol 52: 447–454
Scherer SW, Otulakowski G, Robinson BH, Tsui L-C (1991) Localization of the human dihydrolipoamide dehydrogenase gene (DLD) to 7q31 → q32. Cytogenet Cell Genet 56: 176–177
Schoonen WG, Wanamarta AH, van der Klei-van Moorsel JM, Jakobs C, Joenje H (1990a) Hyperoxia-induced clonogenic killing of HeLa cells associated with respiratory failure and selective inactivation of Krebs cycle enzymes. Mutat Res 237: 173–181
Schoonen WG, Wanamarta AH, van der Klei-van Moorsel JM, Jakobs C, Joenje H (1990b) Respiratory failure and stimulation of glycolysis in Chinese hamster ovary cells exposed to normobaric hyperoxia. J Biol Chem 265: 1118–11124
Schoonen WG, Wanamarta AH, van der Klei-van Moorsel JM, Jakobs C, Joenje H (1991) Characterization of oxygen-resistant Chinese hamster ovary cells. III. Relative resistance of succinate and alpha-ketoglutarate dehydrogenases to hyperoxic inactivation. Free Radic Biol Med 10: 111–118
Sheehan JP, Swerdlow RH, Miller SW, Davis RE, Parks JK, Parker WD, Tuttle JB (1997) Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease. J Neurosci 17: 4612–4622

Sheu KFR, Kim Y-T, Blass JP, Weksler ME (1985) An immunochemical study of pyruvate dehydrogenase deficit in Alzheimer's disease brain. Ann Neurol 17: 444–449
Sheu K-FR, Cooper AJ, Koike K, Koike M, Lindsay JG, Blass JP (1994) Abnormality of the α-ketoglutarate dehydrogenase complex in fibroblasts from familial Alzheimer's disease. Ann Neurol 35: 312–318
Sheu K-FR, Sarkar P, Wasco W, Tanzi RE, Blass JP (1996) A gene locus of dihydrolipoyl succinyltransferase (DLST) is associated with Alzheimer's disease. J Neurochem 66: [Suppl 1] S10B
Sheu K-FR, Brown A, Blass JP (1997) α-Ketoacid dehydrogenase complexes in inborn errors of metabolism and neurodegeneration. J Neurochem 69: S2
Sheu K-FR, Brown AM, Haroutunian V, Kristal BS, Thaler H, Lesser M, Kalaria RN, Relkin NR, Mohs MC, Lilius L, Lannfelt L, Blass JP (1998a) Modulation by DLST of the genetic risk of Alzheimer's disease in a very elderly population. Ann Neurol (in press)
Sheu K-FR, Lilius L, Brown AM, Kristal B, Haratounian V, Mohs R, Relkin N, Kalaria R, Basun H, Wahlund LO, Viitanen M, Lannfelt L, Blass JP (1998b) Polymorphisms of the DLST gene associate with late-onset and with familial Alzheimer's disease. Neurobiol Aging 19(45): 1226 (Abstract)
Simonian SA, Hyman BT (1994) Functional alterations in Alzheimer's disease: selective loss of mitochondrial-encoded cytochrome oxidase mRNA in the hippocampal formation. J Neuropathol Exp Neurol 53: 508–512
Sims NR, Bowen DM, Davison AN (1981) [^{14}C]Acetylcholine synthesis and [^{14}C]carbon dioxide production from [U-^{14}C]glucose by tissue prisms from human neocortex. Biochem J 196: 867–876
Sims NR, Bowen DM, Neary D, Davison AN (1983) Metabolic processes in Alzheimer's disease: adenine nucleotide content and production of $^{14}CO_2$ from [U-^{14}C]glucose in vitro in human neocortex. J Neurochem 41: 1329–1334
Sims NR, Finegan JM, Blass JP (1987a) Altered metabolic properties of cultured skin fibroblasts in Alzheimer's disease. Ann Neurol 21: 509–510
Sims NR, Finegan JM, Blass JP, Bowen DM, Neary D (1987b) Mitochondrial function in brain tissue in primary degenerative dementia. Brain Res 436: 30–38
Small GW, Mazziotta JC, Collins MT, Baxter LR, Phelps ME, Mandelkern MA, Kaplan A, Larue A, Adamson CF, Chang L (1995) Apolipoprotein E type 4 allele and cerebral glucose metabolism in relatives at risk for familial Alzheimer disease. J Am Med Assoc 273: 942–947
Sorbi S, Amaducci L, Blass JP, Bird ED (1982) Pyruvate dehydrogenase complex and choline acetyltransferase in aging and dementia. In: Giacobini E et al. (eds) The again brain: cellular and molecular mechanisms of aging in the nervous system. Raven Press, New York, pp 223–229
Sorbi S, Bird ED, Blass JP (1983) Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. Ann Neurol 13: 72–78
Swerdlow RH, Parks JK, Cassarino DS, Maguire DJ, Aguire RS, Bennett JP, Davis RE, Parker WD (1997) Cybrids in Alzheimer's disease: a cellular model of the disease? Neurol 49: 918–925
Szabo P, Cai X, Ali G, Blass JP (1994) Localization of the gene (OGDH) coding for the E1k component of the α-ketoglutarate dehydrogenase complex to chromosome 7p13–p11.2. Genomics 20: 324–326
Tirmenstein MA, Mathias PI, Snawder JE, Wey HE, Toraason M (1997) Antimony-induced alterations in thiol homeostasis and adenine nucleotide status in cultured cardiac myocytes. Toxicology 119: 203–211
Tomiyama T, Shoji A, Kataoka K, Suwa Y, Asano S, Kaneko H, Endo N (1996) Inhibition of amyloid beta protein aggregation and neurotoxicity by rifampicin. Its possible function as a hydroxyl radical scavenger. J Biol Chem 271: 6839–6844

Van Zuylen AJ, Bosman GJCGM, Ruitenbeck W, Kalmouth PJ, DeGrip WJ (1992) No evidence for reduced thrombocyte cytochrome oxidase in Alzheimer disease. Neurol 42: 1246–1247

Wallace DC, Stugard C, Murdock D, Schurr T, Brown MD (1997) Ancient mtDNA sequences in the human nuclear genome: a potential source of errors in identifying pathogenic mutations. Proc Natl Acad Sci USA 94: 14900–14905

Westergaard N, Sonnewald U, Petersen SB, Schousboe AD (1995) Glutamate and glutamine metabolism in cultured GABAergic neurons studied by ^{13}C NMR spectroscopy may indicate compartmentation and mitochondrial heterogeneity. Neurosci Lett 185: 24–28

Wong-Riley M, Antuono P, Ho KC, Egan R, Hevner R, Liebl W, Huang Z, Rachel R, Jones J (1997) Cytochrome oxidase in Alzheimer's disease: biochemical, histochemical, and immunohistochemical analyses of the visual and other systems. Vision Res 37: 3593–3608

Yates CM, Butterworth J, Tennant MC, Gordan A (1990) Enzyme activities in relation to pH and lactate in post-mortem brain in Alzheimer-type and other dementias. J Neurochem 55: 444–449

Yatin S, Aksenov M, Carney J, Butterfield DA (1997) Alzheimer's amyloid β-peptide (25–35) free radical oxidative modification of neuronal proteins. Neurosci Abstr 23: 634.14

Authors' address: Dr. G. Gibson, Department of Neurology and Neuroscience, Cornell University Medical College, Burke Medical Research Institute, 785 Mamaroneck Avenue, White Plains, NY 10605, USA.

METABOLIC EFFECTS OF DICHLOROACETATE IN PATIENTS WITH DIABETES MELLITUS AND HYPERLIPOPROTEINEMIA

PETER W. STACPOOLE, PH.D., M.D., GEORGE W. MOORE, M.D., AND DAVID M. KORNHAUSER, M.D.

Abstract Dichloroacetate is known to reduce plasma glucose and triglycerides in diabetic and starved animals and to lower plasma lactate under various experimental conditions. To investigate its metabolic effects in man, we administered oral doses (3 to 4 g) of dichloroacetate as the sodium salt to patients with diabetes mellitus or hyperlipoproteinemia or both for six to seven days. Dichloroacetate significantly reduced fasting hyperglycemia an average of 24 per cent ($P<0.01$) from base line and produced marked, concomitant falls in plasma lactate (73 per cent; $P<0.05$ to <0.01) and alanine (82 per cent; $P<0.01$ to <0.001). In addition, it significantly decreased plasma cholesterol (22 per cent; $P<0.01$ to <0.001) and triglyceride (61 per cent; $P<0.01$) levels while increasing (71 per cent; $P<0.01$) plasma ketone-body concentrations. Plasma insulin, free fatty acid and glycerol levels were not affected. Serum uric acid rose, whereas excretion and renal clearance fell. Some patients experienced mild sedation, but no other laboratory or clinical evidence of adverse effects was noted during or immediately after the treatment phase. (N Engl J Med 298:526-530, 1978)

DIABETES mellitus and hyperlipoproteinemia are commonly associated disorders in man, but seldom achieve satisfactory control with current therapy. Pharmacologic intervention traditionally has relied on insulin administration alone or on the combined use of two or more oral medications having selective effects on either carbohydrate or lipid metabolism. A single oral agent that reduces both hyperglycemia and hyperlipoproteinemia without affecting insulin secretion would have obvious clinical potential.

In 1962, Vailati and Rabassini[1] briefly described the use of dichloroacetate administered as the di-isopropylammonium salt in the short-term treatment of human diabetes mellitus. The effects of dichloroacetate on various aspects of intermediary metabolism have been studied extensively in several animal models. The drug reduces blood sugar levels in both diabetic[2-4] and starved[5] animals but not in healthy, fed animals. In addition, dichloroacetate lowers plasma triglyceride levels and hepatic lipid content in diabetic rats.[6] None of the drug's effects on carbohydrate or fat metabolism in animals are mediated through actions on insulin secretion (unpublished data) or intestinal glucose transport.[4]

In the present study, we administered the sodium salt of dichloroacetate by mouth to patients with diabetes or hyperlipoproteinemia (or both). The drug decreased fasting hyperglycemia and induced profound falls in plasma lactate and alanine levels. In addition, it markedly reduced circulating cholesterol and triglyceride while producing a moderate rise in plasma ketone bodies.

From the Diabetes-Endocrinology Research Center and the divisions of Endocrinology and Clinical Pharmacology, Department of Medicine, Vanderbilt University School of Medicine (address reprint requests to Dr. Stacpoole at the Department of Medicine, Vanderbilt University Hospital, Nashville, TN 37232).

Presented in part at the annual meeting of the American Diabetes Association, San Francisco, CA, June 19-23, 1976.

Supported in part by research grants (GM-15431, 5-M01-RR 95, AM 17076 and AM 19587) from the National Institutes of Health (Dr. Moore is an endocrinology fellow sponsored by the U.S. Air Force, and Dr. Kornhauser was the recipient of a fellowship for careers in clinical pharmacology from the Pharmaceutical Manufacturers Association Federation).

MATERIALS AND METHODS

Subjects

Patients ranged in age between 42 and 71 years. Each had non-ketotic diabetes mellitus either alone (fasting plasma glucose >130 mg per deciliter) or combined with hyperlipoproteinemia (hypercholesterolemia or hypertriglyceridemia or both). Weights varied from 108 to 154 per cent of ideal values based on Metropolitan Life Insurance statistics.[7]

The study was divided into two phases. The first, making up the initial part, consisted of seven female patients: six had fasting hyperglycemia, and six had elevated plasma cholesterol or triglyceride or both. The second phase consisted of three female patients and one male patient who were more extensively investigated. Each of these subjects had both fasting hyperglycemia and hyperlipoproteinemia. No patients had received treatment other than diet for their diabetes or hyperlipoproteinemia for at least 10 days before study. Patients received no medications except mild analgesics for other medical problems throughout the course of this investigation.

This investigation was approved by the Vanderbilt University Hospital Human Experimentation Committee and by the Food and Drug Administration (IND #11,145). Informed consent was obtained from each patient before study.

Procedures

Studies were performed while the patients were in the Clinical Research Center at Vanderbilt University Hospital. Each patient received a constant diet isocaloric with his or her normal, outpatient regimen. After an in-hospital period of dietary equilibration lasting for 10 to 14 days for the first phase and 18 to 22 days for the second, dichloroacetate was administered as a single, daily, oral dose of either 3 or 4 g for six to seven days. After therapy, patients were studied for an additional period of seven days in the first phase and eight to 15 days in the second phase.

Serial analyses of the following measurements were made throughout the study: complete blood count with differential, platelet count, reticulocyte count, SMA-6 and 12 or SMAC, prothrombin time, partial thromboplastin time and urinalysis. Electrocardiograms were obtained before, during and after drug treatment.

Fasting blood samples were obtained 15 hours after the patient's last meal; this schedule corresponded to 10 hours after each dose of dichloroacetate. After centrifugation at 4°C, plasma was collected for analysis. Glucose,[8] lactate,[9] β-hydroxybutyrate[10] and glycerol[11] were measured by standard enzymatic technics. We measured individual plasma amino acids with a Technicon NCZP Amino Acid analyzer (Technicon Corporation, Tarreytown, New Jersey). Alanine was determined by column chromatography.[12] Free fatty acids

were estimated by radiochemical assay,[13] and cholesterol and triglyceride by colorimetric[14] and fluorimetric methods, respectively. Insulin levels were determined by radioimmunoassay (Phadebas). Plasma lipoprotein classes with d = 1.006 (very-low-density lipoproteins), d<1.006 to 1.063 (low-density lipoproteins) and d<1.063 to 1.210 (high-density lipoproteins) were isolated by ultracentrifugation after adjustment of the density of the plasma with solid sodium bromide. Total lipids, extracted from the isolated lipoproteins with chloroform:methanol (2:1 vol/vol), were separated into lipid classes by thin-layer chromatography (Silical Gel G). Cholesterol and cholesterol esters, triglyceride and phospholipid were determined on the appropriate band.[16] Twenty-four-hour urine samples were collected for measurement of sodium, potassium, creatinine, glucose and uric acid. Aliquots of urine were taken before each meal for determination of glucose (Clinitest) and ketones (Acetest).

Chemicals

Sodium dichloroacetate was purchased from Tokyo Kasei Kogyo Company, Limited, Tokyo, Japan. We established its purity by means of gas-liquid chromatography. Gelatin capsules containing 250 mg or 500 mg of dichloroacetate with lactose filler were manufactured by the Vanderbilt Hospital Pharmacy.

Analysis

In the second phase with each patient used as his own control, the paired Student t-test was employed to determine statistical significance between the mean of the three "control" values immediately before administration of the drug and the mean of the values obtained during the final three days of drug therapy.

Results

First Phase

Table 1 shows the means of fasting plasma concentrations of glucose, lactate, triglyceride and cholesterol for the three days immediately before dichloroacetate therapy ("control") and the final three days of dichloroacetate treatment ("DCA").

The drug reduced fasting blood sugar in all seven patients studied. The higher the inital degree of hyperglycemia, the greater the fall in blood sugar during

Table 1. Effect of Dichloroacetate (DCA) on Fasting Plasma Glucose, Lactate and Lipids in Patients with Diabetes or Hyperlipoproteinemia (or Both).

Patient No.	Glucose mg/dl		Lactate mmol/liter		Cholesterol mg/dl		Triglyceride mg/dl	
	control*	DCA†	control	DCA	control	DCA	control	DCA
1	400	176			260	280	1,000	640
2	265	205	1.6	0.6	215	170	187	62
3	137	104			163	116	149	64
4	214	194	2.3	0.9	290	257	125	48
5	154	132	1.8	1.5	268	236	96	78
6	106	97			215	170	237	120
7	158	105			216	171	233	160

*Mean values of 3 days immediately before onset of therapy.
†Mean values of final 3 days of therapy.

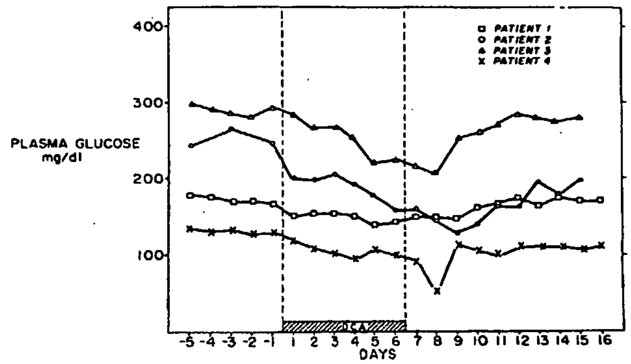


Figure 1. Effect of Dichloroacetate (DCA) on Fasting Plasma Glucose (Broken Vertical Lines Indicate Period of Treatment).

dichloroacetate administration — a finding consistent with previous animal studies.[3,4] Fasting plasma lactate concentrations were reduced in the three patients in whom they were measured.

Plasma cholesterol rose in Patient 1 but fell in the remaining subjects during drug testing. More impressive than its effects on cholesterol, however, was the often striking reduction by dichloroacetate of plasma triglyceride levels. Every patient, regardless of baseline triglyceride values, experienced some degree of triglyceride lowering. In general, the higher the pretreatment level, the greater the reduction occurring during therapy.

Second Phase

On the basis of these initial results, we studied in greater detail the metabolic effects of dichloroacetate in four additional subjects with fasting hyperglycemia and hyperlipoproteinemia. Figures 1 to 4 summarize the results obtained beginning five days before treatment and continuing through the completion of the study.

All subjects responded with a significant fall ($P<0.01$ for each patient) in fasting blood sugar (Fig. 1). The magnitude of the reduction was accentuated by the rebound in blood sugar when drug therapy was stopped. The maximum reduction in blood sugar of approximately 120 mg per deciliter was achieved in Patient 2, with the blood sugar nadir occurring two days after therapy was ended. In this person, fasting blood sugar remained below control levels even eight days after the final day of treatment.

Plasma lactate and alanine (Fig. 2) were also measured in these patients. Although all subjects had normal or slightly elevated blood lactate levels before treatment, they showed marked falls ($P<0.05$ to $P<0.01$) in plasma lactate during dichloroacetate administration, beginning within 10 hours after the first dose and reaching lows of 0.2 to 0.4 mmol per liter (up to a 90 per cent decrease from pretreatment levels).

These reductions in lactate persisted several days after therapy. Likewise, plasma alanine dropped precipitously ($P<0.01$ to $P<0.001$) from 0.45 to 0.60 mmol per liter before therapy to about 0.060 mmol per liter. As with lactate, plasma alanine remained well below control levels for several days after treatment was stopped. Plasma levels of all other amino acids were not significantly changed during the treatment phase (results not shown).

Plasma cholesterol (Fig. 3) did not change ($P>0.05$) in Patient 1 but fell significantly ($P<0.01$ to $P<0.001$) in the other three during administration of

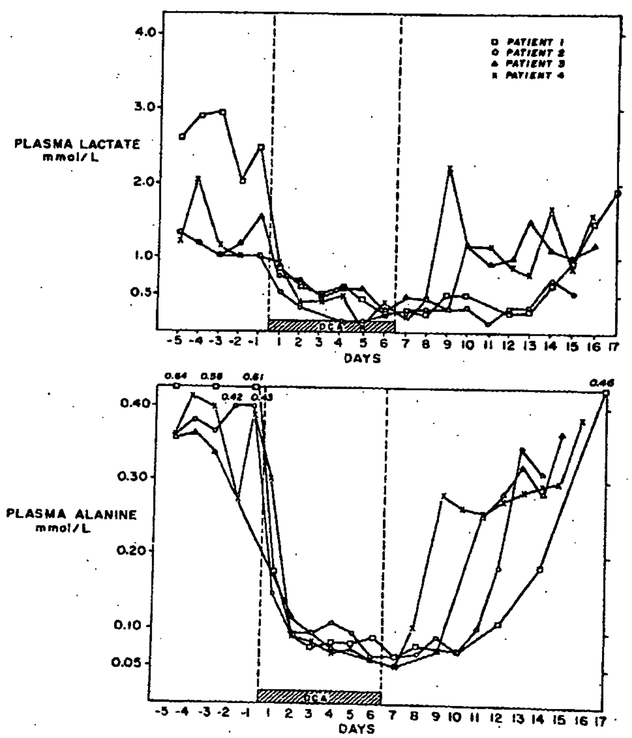


Figure 2. Effect of Dichloroacetate (DCA) on Fasting Plasma Lactate and Alanine (Broken Vertical Lines Indicate Period of Treatment).

dichloroacetate. In Patient 3 cholesterol had fallen approximately 90 mg per deciliter by the third post-treatment day, and was still below control levels nine days after therapy had ended.

All subjects showed marked ($P<0.01$) reductions in plasma triglyceride (Fig. 3) during and after dichloroacetate administration. In Patient 1, whose cholesterol did not change during drug treatment, pretreatment triglycerides averaged approximately 1000 mg per deciliter. Three days after the first dose of dichloroacetate, plasma triglyceride had dropped to 490 mg per deciliter and continued to fall to a low of 160 mg per deciliter by the seventh day. After cessation of therapy, plasma triglycerides increased slowly but,

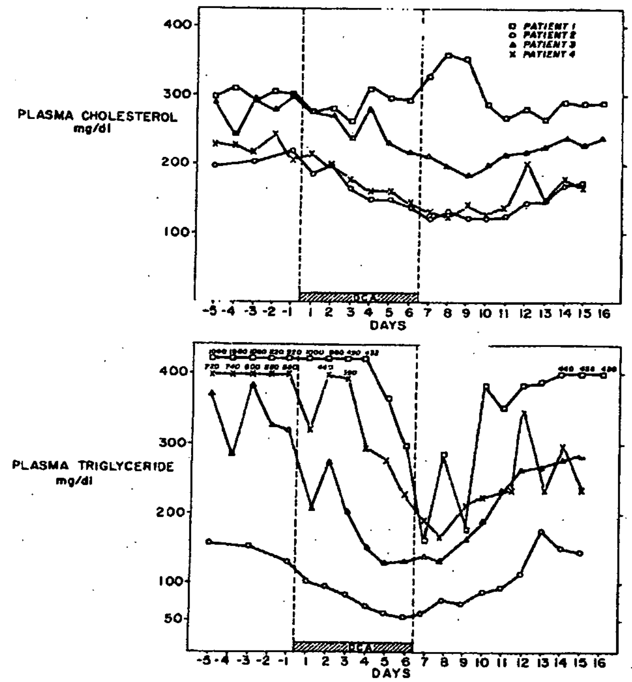


Figure 3. Effect of Dichloroacetate (DCA) on Fasting Plasma Cholesterol and Triglycerides (Broken Vertical Lines Indicate Period of Treatment).

throughout the remaining nine days of hospitalization, never rose above 488 mg per deciliter. Similarly, the triglycerides of Patient 3 fell sharply during therapy and did not achieve control levels during eight days of post-treatment observation.

The very-low-density-lipoprotein fraction was the lipoprotein component most significantly affected by dichloroacetate (results not shown). Each of the four patients studied registered falls in very-low-density-lipoprotein triglyceride, phospholipid, cholesterol and

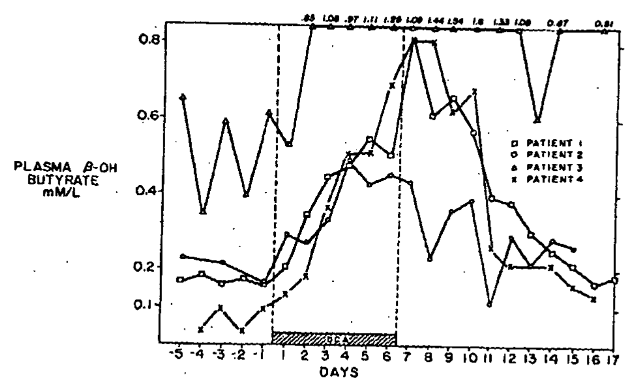


Figure 4. Effect of Dichloroacetate (DCA) on Fasting Plasma β-Hydroxybutyrate (Broken Vertical Lines Indicate Period of Treatment).

cholesterol esters; to a lesser extent, triglyceride was also reduced. In Patient 1, the only subject of the second phase who failed to show a fall in plasma cholesterol with dichloroacetate therapy, low-density-lipoprotein cholesterol and cholesterol esters increased slightly during drug administration.

Plasma free fatty acids and glycerol, measured throughout the study (results not shown), were not appreciably affected by therapy. In contrast, plasma β-hydroxybutyrate levels (Fig. 4) rose significantly ($P<0.01$) in all four subjects during drug treatment. In Patients 1 and 2, these changes were accompanied by the development of ketonuria, indicating a concomitant increase in acetoacetate concentration. No significant changes in serum bicarbonate levels, however, occurred in any subject.

Table 2. Effect of Dichloroacetate (DCA) on Uric Acid Metabolism in Four Patients.

Period*	Uric Acid		Urate Clearance
	serum	urine	
	mg/dl	mg/24 hr	ml/min
PRE	6.8±1.0†	548±61	6.2±1.7
DCA	10.4±0.9	420±43	3.0±0.5
POST	6.6±1.0	639±96	6.2±1.5

*Values represent those obtained 1 day before (PRE), the final day of & the 7th day after cessation of (POST) administration of drug.

†Mean ± SEM.

Throughout the entire study, patients maintained relatively stable body weight (average weight change, ±0.7 kg over four to five weeks) and normal fasting insulin levels, which did not change significantly during dichloroacetate administration.

Few adverse affects were noted in association with treatment. Approximately half the subjects in both phases experienced mild sedation during the period of drug testing. In addition, in the four patients of the second phase, serum uric acid levels had increased approximately 35 per cent by the last day of administration (Table 2). This change was accompanied by a fall in both urate excretion and urate clearance. Within a week after therapy was stopped, however, serum and urinary uric acid levels and urate clearance returned to their pretreatment values.

No other laboratory indexes (including blood counts and tests of liver and kidney function) were altered during or after drug administration.

Discussion

These investigations demonstrate that dichloroacetate is capable of eliciting noteworthy metabolic effects in man. The earliest and most striking changes observed were in plasma lactate and alanine concentrations. The reduction in the levels of these gluconeogenic substrates antedate the fall in fasting blood sugar and persist beyond the period of drug administration. These findings are consistent with animal studies[17-20] showing that dichloroacetate stimulates pyruvate dehydrogenase in peripheral tissues, thereby enhancing pyruvate oxidation to acetyl coenzyme A. Such an effect would tend to shunt more lactate and alanine into carbon dioxide formation and provide less substrate for hepatic glucose synthesis. Other evidence in animals, however, suggests that dichloroacetate may lower blood sugar by facilitating peripheral glucose oxidation[3,22] and by inhibiting hepatic glucose synthesis from three-carbon precursors.[23] No single mechanism may be sufficient, therefore, to account for the reduction in hyperglycemia produced by administration of the drug.

The mechanism by which dichloroacetate induces a rapid and sustained fall in plasma lipids, particularly triglycerides, is unknown. The decrease in triglycerides probably cannot be attributed to a reduction in circulating fatty acids. Moreover, there are no animal or human studies to suggest that short-term administration of the drug enhances hepatic triglyceride stores.[6,24,25] It is possible, however, that the drug stimulates the oxidation of triglycerides by the liver to ketones. Although the quantitative importance of such a mechanism cannot be determined by present data, it could account for the steady rise in plasma β-hydroxybutyrate and urinary acetoacetate occurring along with the progressive fall in plasma triglycerides. If this mechanism were operative, one might expect a subsequent decline in ketone-body production once the maximum reduction in circulating triglycerides was achieved by dichloroacetate. Animal studies,[21,26] however, have shown that the drug inhibits uptake and utilization of ketone bodies by peripheral tissues, and this effect may serve to maintain elevated plasma ketone levels independently of changes in plasma triglycerides. In addition, although plasma insulin levels did not change appreciably during drug treatment, plasma glucagon levels were not measured. It is conceivable that a rise in the glucagon/insulin ratio occurred and accounted, at least in part, for the increase in plasma ketone bodies observed. No primary effect of dichloroacetate on cholesterol metabolism is known. The reduction in plasma cholesterol seen in the majority of the patients may have been secondary to the fall in plasma very-low-density lipoproteins.

It is not known why some patients experienced mild sedation during the period of drug therapy. To our knowledge, dichloroacetate has no other effects on central-nervous-system function. The mechanism by which the drug elevates serum uric acid levels is likewise unclear; however, this change occurs concomitantly with a reduction in urinary uric acid excretion and therefore in renal clearance. It may be that urate excretion is competitively inhibited, either by the dichloroacetate anion itself or by the higher

blood levels of the negatively charged ketone bodies that occur during its administration.

The minimally effective maintenance doses and optimal therapeutic regimens necessary to produce and sustain the effects on blood sugar and lipids have not yet been determined. In addition, the consequences of prolonged, statistically significant reductions in the key gluconeogenic substrates, alanine and lactate, particularly during periods of prolonged fasting, require further scrutiny. Of particular interest is the ability of dichloroacetate to lower blood lactate levels rapidly. In vivo animal studies have demonstrated that the drug both prevents and reverses lactic acidosis induced by phenformin administration, exercise or epinephrine infusion.[26-30] These findings suggest that it may be beneficial in the treatment of lactic acidosis in human beings. The activation of pyruvate dehydrogenase and subsequent stimulation of pyruvate and lactate oxidation by dichloroacetate seems to offer a safe, physiologic mechanism by which lactate accumulation could be controlled.

Although we recognize certain therapeutic implications derived from this study, we must emphasize the preliminary nature of our investigation in that only a few patients were treated for a very brief time. The efficacy and safety of chronic dichloroacetate administration are unknown. Further studies are required to answer these questions and to elucidate the mechanisms by which dichloroacetate exerts its metabolic effects in man.

We are indebted to Dr. William W. Lacy for the plasma amino acid measurements, to Dr. Henry G. Wilcox for the lipoprotein fractionation, to Dr. Ulrich Keller for the plasma free fatty acid and glycerol determinations and to Drs. David Rabinowitz, Oscar B. Crofford and John A. Oates for continued support.

References

1. Vailati G, Rabassini A: Sull'azione impoglicemizzante del dicloroetanotato di diisopropilammonio nell'uomo. Riv Crit Clin Med (Firenze) 62:105-109, 1962
2. Lorini M, Ciman M: Hypoglycaemic action of di*iso*propylammonium salts in experimental diabetes. Biochem Pharmacol 11:823-827, 1962
3. Stacpoole PW, Felts JM: Diisopropylammonium dichloroacetate (DIPA) and sodium dichloroacetate (DCA): effect on glucose and fat metabolism in normal and diabetic tissue. Metabolism 19:71-78, 1970
4. Eichner HL, Stacpoole PW, Forsham PH: Treatment of streptozotocin diabetes with di-isopropylammonium dichloroacetate (DIPA). Diabetes 23:179-182, 1974
5. Blackshear PJ, Holloway PAH, Alberti KGMM: The metabolic effects of sodium dichloroacetate in the starved rat. Biochem J 142:279-286, 1974
6. Felts JM, Staprans I, Stacpoole PW: The effect of sodium dichloroacetate (DCA) on plasma triglycerides of streptozotocin diabetic rats. Diabetes 25:Suppl 1:363, 1976
7. Fifty years of life conservation. Stat Bull Metropol Life Ins Co 40:1-4, 1959
8. Slein MW: D-glucose, Determination with hexokinase and glucose-6-phosphate dehydrogenase, Methods of Enzymatic Analysis. Edited by H-U Bergmeyer. New York, Academic Press, 1963, pp 117-123
9. Hohorst H-J: L-(+)-Lactate, Determination with lactic dehydrogenase and DPN, Methods of Enzymatic Analysis. Edited by H-U Bergmeyer. New York, Academic Press, 1963, pp 266-270
10. Williamson DH, Mellanby J: D-(−)-β-hydroxybutyrate, Methods of Enzymatic Analysis. Edited by H-U Bergmeyer. New York, Academic Press, 1963, pp 459-461
11. Wieland O: Glycerol, Methods of Enzymatic Analysis. Edited by H-U Bergmeyer. New York, Academic Press, 1963, pp 211-214
12. Chiasson JL, Liljenquist JE, Sinclair-Smith BC, et al: Gluconeogenesis from alanine in normal postabsorptive man. Diabetes 24:574-584, 1975
13. Ho RJ: Radiochemical assay of long-chain fatty acids using ^{63}Ni as tracer. Anal Biochem 36:105-113, 1970
14. Levine JB, Zak B: Automated determination of serum total cholesterol. Clin Chim Acta 10:381-384, 1964
15. Kessler G, Lederer H: Fluorometric measurement of triglycerides, Automation in Analytical Chemistry [1965]. Edited by LT Skeggs Jr. New York, Mediad, 1966, pp 341-344
16. Wilcox HG, Dunn GD, Heimberg M: Effects of several common long chain fatty acids on the properties and lipid composition of the very low density lipoprotein secreted by the perfused rat liver. Biochim Biophys Acta 398:39-54, 1975
17. Whitehouse S, Randle PJ: Activation of pyruvate dehydrogenase in perfused rat heart by dichloroacetate. Biochem J 134:651-653, 1973
18. Whitehouse S, Cooper RH, Randle PJ: Mechanism of activation of pyruvate dehydrogenase by dichloroacetate and other halogenated carboxylic acids. Biochem J 141:761-774, 1974
19. Kerbey AL, Randle PJ, Cooper RH, et al: Regulation of pyruvate dehydrogenase in rat heart. Biochem J 154:327-348, 1976
20. Mapes JP, Harris RA: Regulatory function of pyruvate dehydrogenase and the mitochondrion in lipogenesis. Lipids 10:757-764, 1976
21. McAllister A, Allison SP, Randle PJ: Effects of dichloroacetate on the metabolism of glucose, pyruvate, acetate, 3-hydroxybutyrate and palmitate in rat diaphragm and heart muscle *in vitro* and on extraction of glucose, lactate, pyruvate and free fatty acids by dog heart *in vivo*. Biochem J 134:1067-1081, 1973
22. Searle G, Felts JM, Shakelford R, et al: Effects of dichloroacetate in the depancreatized dog: glucose pool, space, turnover, and oxidation. Diabetes 25:Suppl 1:385, 1976
23. Stacpoole PW: Effect of dichloroacetate on gluconeogenesis in isolated rat hepatocytes. Metabolism 26:107-116, 1977
24. *Idem:* Review of the pharmacologic and therapeutic effects of diisopropylammonium dichloroacetate (DIPA). J Clin Pharmacol 9:282-291, 1969
25. Blackshear PJ, Holloway PAH, Alberti KGMM: Metabolic interactions of dichloroacetate and insulin in experimental diabetic ketoacidosis. Biochem J 146:447-456, 1975
26. Holloway PAH, Alberti KGMM: Reversal of phenformin-induced hyperlactaemia by dichloroacetate in normal and diabetic rates. Diabetologia 11:350-351, 1975
27. *Idem*: Phenformin-induced lactic acidosis: prevention by dichloroacetate. Clin Sci Mol Med 50:33p, 1976
28. Loubatières A, Ribes G, Valette G, et al: Correction de l'hyperlactatémie et de l'acidose lactique experimentales par le dichloroacetate de sodium. C R Acad Sci [D] (Paris) 283:1125-1127, 1976
29. Loubatières AL, Ribes G, Valette G: Pharmacological agents and acute experimental hyperlactaemia in the dog. Br J Pharmacol 58:429, 1976
30. *Idem:* Réduction par le dichloroacétate de sodium et par l'insuline des hyperlactatémies graves consecutives chez le Chien anesthésié ou éveillé à l'administration de phenformine. C R Acad Sci [D] (Paris) 284:325-327, 1977